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Inhibition of Bound Enzymes. I. Antienergistic Interaction of Chemical and Diffusional Inhibition†

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ABSTRACT: When the diffusion of the substrate to bound enzymes is relatively slow the substrate concentration is lower in the microenvironment than in the macroenvironment and the resulting reduction in the enzyme activity is referred to as diffusional inhibition. On the other hand, an inhibitor, which reduces the enzyme activity *per se*, attenuates the magnitude of diffusional inhibition. Because of this antienergistic interaction between chemical and diffusional inhibition the activity of the bound enzyme at steady state is less affected by an inhibitor in the presence than in the absence of diffusion limitations for the substrate. As diffusional inhibition frequently occurs in heterogeneous enzyme systems, the antienergism has to be taken into account when the action of inhibitors is considered on enzymes artificially immobilized or bound to membranes in the cellular milieu.

The inhibition of enzymes has been extensively treated in the literature (Webb, 1963) because inhibitors are valuable tools in the biological sciences for the study of isolated enzymes and of the various aspects of cellular metabolism. They also play a well-established regulatory role in living systems. Most studies have been concerned with the inhibition of enzymes in free solution and little attention has been paid to the action of inhibitors on enzymes in heterogeneous media. The cellular milieu, however, is compartmented and most enzymes are embedded in or bound to membranes (Greville, 1969; Lehninger, 1970). In such heterogeneous systems the rate of the reaction is usually attenuated by the relative slowness of the substrate diffusion to the enzyme (Engasser and Horvath, 1973) and this phenomenon is referred to as diffusional inhibition. As a result the effect of an inhibitor on the enzymic reaction, *i.e.*, chemical inhibition, in heterogeneous media manifests itself in a way different from that in a free solution where diffusional resistances are negligible.

In this study the interplay between diffusional and chemical inhibition is treated quantitatively. Both external and internal diffusion resistances are considered (Horvath and Engasser, 1974), and it is assumed that the noncompetitive inhibitor is neither a substrate nor a product of the enzymic reaction. The results shed light on the inhibition of naturally and artificially immobilized enzyme systems.

Theoretical Analysis

Inhibition with External Diffusion Resistance. When the enzyme is attached to a surface and the substrate is transported from the macroenvironment to the enzyme through an "unstirred layer" of liquid or across a membrane, the external diffusion resistance in the nonreactive medium may result in a depletion of the substrate at the enzymic surface. Then the surface concentration of the substrate in the microenvironment of the bound enzyme, $[S_0]$, is lower than that in the macroenvironment, [S].

For Michaelis-Menten kinetics the rate of enzymic reaction, v, with noncompetitive inhibition is given by

$$v = \frac{V_{max}[S_0]}{[1 + ([I]/K_I)][K_M + [S_0]]} \quad (1)$$

where V_{max} is the saturation rate in the absence of the inhibitor, [I] is the concentration of the inhibitor, and K_M and K_I are the Michaelis constant and inhibition constant, respectively. It is assumed that the inhibitor concentration is uniform throughout the system.

In most cases the transport of substrate to the reactive surface can be expressed by the product of a transport coefficient, h_s, and the driving force given by the difference between the substrate concentration in the macro- and the microenvironment, $([S] - [S_0])$. At steady state both the consecutive transport and consumption of the substrate by the chemical reaction

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‡ Recipient of Yale University Fellowship.

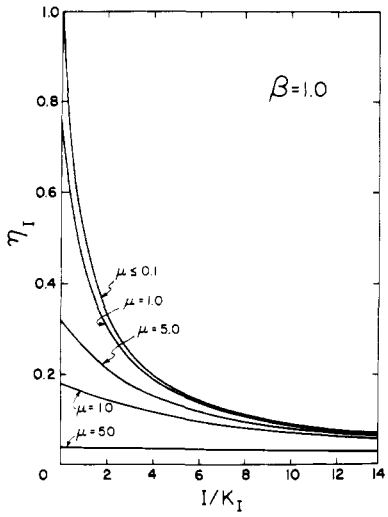

FIGURE 1: Graph illustrating the effect of a noncompetitive inhibitor on the activity of bound enzymes with external diffusion of the substrate. The efficiency factor, η_I, which expresses the combined effect of chemical and diffusional inhibition, is plotted as a function of the normalized inhibitor concentration, $[I]/K_I$, at different values of the substrate modulus, μ, *i.e.*, at different magnitudes of the external diffusion resistance.

proceed at the same rate so that

$$h_s([S] - [S_0]) = \frac{V_{max}[S_0]}{[1 + ([I]/K_I)][K_M + [S_0]]} \quad (2)$$

By defining a dimensionless substrate concentration, β, as

$$\beta = [S]/K_M \quad (3)$$

and the dimensionless substrate modulus, μ, in the absence of an inhibitor as

$$\mu = V_{max}/h_s K_M \quad (4)$$

eq 2 can be written in dimensionless form as

$$\beta - \beta_0 = \frac{\mu\beta_0}{[1 + ([I]/K_I)][1 + \beta_0]} \quad (5)$$

In order to analyze the interaction between chemical and diffusional inhibition, first the surface concentration is evaluated by eq 5, then the enzymic activity is calculated according to eq 1 for different values of the substrate concentration, β, the modulus, μ, and the dimensionless inhibitor concentration, $[I]/K_I$.

Efficiency Factor. The combined effect of chemical and diffusional inhibition is conveniently expressed by the efficiency factor with inhibition, η_I, which is defined as the ratio of the effective enzymic activity, v, to the activity of the enzyme in the absence of both kinds of inhibition. Then the following relationship holds.

$$v = \eta_I V_{max}[S]/(K_M + [S]) \quad (6)$$

In the absence of diffusional inhibition the efficiency factor is simply given by $[1 + ([I]/K_I)]^{-1}$, as seen from eq 1. Thus, the magnitude of the chemical inhibition is expressed by the factor $[1 + ([I]/K_I)]$.

On the other hand, in the absence of chemical inhibition η_I is equal to the so-called effectiveness factor, η, which has been extensively treated in the literature (Aris, 1969). Under such conditions at a given substrate concentration, [S], the magnitude of the diffusional inhibition is expressed by the factor $1/\eta$ and has been shown to be dependent only on the substrate modulus, μ (Horvath and Engasser, 1974). Then the value of μ, which is defined in eq 4 as the ratio of the first-order rate constant of the enzymic reaction to the substrate transport coefficient, determines the relative contributions of the transport and kinetic parameters to the effective reaction rate. At small values of μ ($\mu < 0.1$), the inherent enzymic activity at the surface is sufficiently low or the inherent transport rate of the substrate is sufficiently fast to prevent any significant depletion of substrate at the surface so that no diffusional inhibition occurs and η is close to unity. At very large values of μ the surface is so active that $[S_0]$ approaches zero. Under these conditions the activity of the bound enzyme is practically equal to the maximum rate of molecular and convective diffusion of the substrate and essentially independent of the kinetic parameters of the enzymic reaction. Then the effectiveness factor is in effect inversely proportional to μ.

When both chemical and diffusional inhibition occur the efficiency factor depends on both μ and $[I]/K_I$. The combined effect of chemical and diffusional inhibition is shown in Figure 1, where η_I is plotted as a function of $[I]/K_I$ for different values of μ at $\beta = 1$. At a constant β the variation of η_I also represents the variation of the enzymatic activity since the rate of reaction is proportional to η_I as seen from eq 6. The curve for $\mu \leq 0.1$ in Figure 1 shows the intrinsic dependence of the enzymic activity on the dimensionless inhibitor concentration, $[I]/K_I$, because at such low values of μ diffusional inhibition is absent. At higher values of μ, that is, with increasing diffusional inhibition, the overall activity decreases less with increasing inhibitor concentration and at $\mu = 50$, the activity is practically independent of $[I]/K_I$, at least in the domain investigated. Consequently, at high inhibitor concentrations, the different curves converge to that obtained in the absence of substrate depletion.

Antienergism and Effective Substrate Modulus. Figure 1 clearly shows that when chemical and diffusional inhibition are acting simultaneously, their combined effect in decreasing the rate of reaction is smaller than the sum of the effects that would be obtained if the two inhibition phenomena were acting independently. This antienergism, which is the opposite o synergism, can be qualitatively interpreted as follows. Substrate depletion at the enzymic surface occurs when the inherent diffusion rate is slow with respect to the inherent enzymic activity. An enzyme inhibitor that decreases the inherent enzymic activity also reduces the relative slowness of substrate transport. Thus chemical inhibition in heterogeneous enzyme systems can be characterized by two antagonistic effects acting simultaneously on the rate of reaction: the decrease of the inherent enzyme activity and the reduction of the degree of diffusional inhibition.

This antienergistic interaction is quantitatively expressed by introducing the effective substrate modulus, μ_I, which has the same physical meaning for the chemically inhibited system as that of μ in the absence of an inhibitor. Since the maximum rate of the enzymic reaction is reduced by the factor $[1 + ([I]/K_I)]$ in the presence of a noncompetitive inhibitor the effective substrate modulus, in view of eq 4, is defined by

$$\mu_I = \frac{\mu}{1 + ([I]/K_I)} \quad (7)$$

Equation 7 shows that an enzyme-inhibitor reduces the effective substrate modulus, and consequently the relative magnitude of diffusional inhibition. At sufficiently large inhibitor concentrations diffusional inhibition vanishes when μ_I becomes smaller than 0.1.

It is noted that at high values of μ, as shown in Figure 1 for $\mu = 50$, the effective enzymic activity is unaffected by the increasing inhibitor concentration. This insensitivity of a system

with high transport resistances to chemical inhibition is due to the fact that when η_I is large the magnitude of diffusional inhibition is proportional to μ_I for all practical reasons, therefore, inversely proportional to $[1 + ([I]/K_I)]$. Since the magnitude of chemical inhibition is equal to this quantity the two kinds of inhibition cancel out each other.

Inhibition with Internal Diffusional Resistance. When enzymes are embedded in a porous medium like a membrane or cross-linked to form an insoluble protein the substrate has to diffuse from the surface into the enzymic medium in order to react at the catalytic sites. Under such conditions diffusion and chemical reaction are taking place in parallel and as a result the mathematical treatment of the overall reaction is more complex than for external transport when diffusion and reaction occur in series (Aris, 1969).

A previous study (Engasser and Horvath, 1973) has shown that in the absence of chemical inhibition the extent of diffusional inhibition depends, at a given substrate macroenvironmental concentration, on the pertinent substrate modulus, ϕ, defined as

$$\phi = l(V_{max}'''/K_M D_s)^{1/2} \quad (8)$$

where l is the membrane thickness, V_{max}''', the saturation reaction rate per unit volume of porous medium, and D_s is the substrate diffusivity inside the membrane. The combined effect of chemical and diffusional inhibition is again expressed by the pertinent efficiency factor, η_I, which is defined by eq 6.

With internal diffusion the dependence of η_I on $[I]/K_I$ is very similar to that with external diffusion, as shown in Figure 2 for different values of ϕ at $\beta = 1$. Due to the antienergistic interaction between the two types of inhibition the increasing inhibitor concentration has a smaller effect on the overall activity when diffusional inhibition occurs at $\phi > 1$ than in its absence at $\phi \leq 1$. The inhibitor not only reduces the inherent activity of the enzyme by a factor of $[1 + ([I]/K_I)]$, but also diminishes the extent of substrate depletion, which is conveniently characterized for internal diffusion by ϕ_I, the effective substrate modulus. It is given by

$$\phi_I = \phi/[1 + ([I]/K_I)]^{1/2} \quad (9)$$

The physical significance of ϕ_I is analogous to that of μ_I given in eq 7.

In contrast to the external diffusion controlled reaction the enzymic activity decreases with increasing inhibitor concentration even at large values of ϕ. Comparing the denominators in eq 7 and 9 one can easily explain this result in view of the previous discussion.

Discussion

The preceding analysis has demonstrated that the overall activity of a bound enzyme is less affected by a noncompetitive inhibitor in the presence than in the absence of diffusional resistances for the substrate. This phenomenon has been explained by the antienergistic interplay of diffusional and chemical inhibition, that is, by the attenuation of the magnitude of the diffusional inhibition in the presence of an enzyme inhibitor. The concept is not restricted to noncompetitive inhibitors but is applicable to all kinds of inhibitors and even to enzyme denaturation (Ollis, 1972). Its practical consequences can be far reaching with regard to both immobilized enzyme technology and cellular physiology.

For example, Carlsson *et al.* (1972) found that the rate of hydrolysis of *N*-acetyl-L-tyrosine ethyl ester by chymotrypsin immobilized on Sephadex particles was inhibited by *N*-acetyl-D-tryptophan and *N*-acetyl-D-tryptophan methyl ester to a markedly lesser extent than both the soluble and solubilized chymotrypsin-Sephadex conjugates. Similar results were reported for the inhibitory effect of benzamidine on the hydrolysis of *N*-benzoyl-L-arginine ethyl ester by trypsin bound to Sephadex. The curves presented by the authors for the dependence of the enzyme activity on the inhibitor concentration are similar to those shown in Figures 1 and 2 and demonstrate the antienergism of diffusional and chemical inhibition.

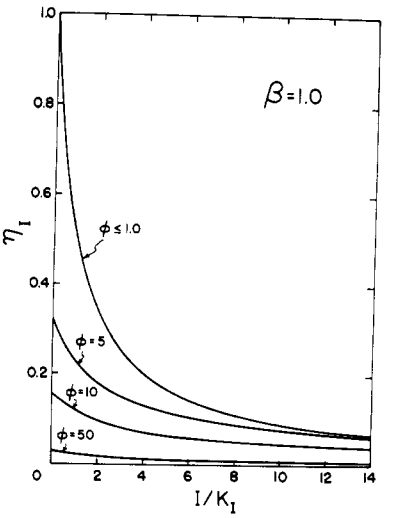

FIGURE 2: Graph illustrating the effect of a noncompetitive inhibitor on the activity of bound enzymes with internal diffusion of the substrate. The efficiency factor, η_I, which expresses the combined effect of chemical and diffusional inhibition, is plotted as a function of the normalized inhibitor concentration, $[I]/K_I$, at different values of the substrate modulus, ϕ, *i.e.*, at different magnitudes of the internal diffusion resistance.

The magnitude of both inhibition and denaturation of bound enzymes may be underestimated by the experimentator when the measured rate of reaction is significantly affected by diffusion, and both phenomena may be completely masked in the case of strong diffusional resistances. It is therefore advisable to establish experimentally that diffusion limitations do not affect the rate of reaction with the heterogeneous enzyme system under investigation before any statement is made about the effect of chemical inhibition or the stability of the bound enzyme. As shown earlier (Horvath and Engasser, 1974), the Eadie–Hofstee types of plots are particularly useful for the diagnosis of external or internal diffusional resistances.

The antienergism between diffusional and chemical inhibition described in this study applies also to living systems, provided the magnitude of diffusion resistances is sufficiently large. The attenuating effect of "unstirred layers" on the rate of uptake of different species across biological membranes has been well established (Naftalin, 1971; Wilson *et al.*, 1971; Hersey and High, 1972; Zander and Schmid-Schönbein, 1972). Very little is known, however, about the magnitude of intracellular diffusional resistances and their role in metabolic processes. Therefore the importance of diffusional effects on enzymic reactions in the cellular milieu has to be approximated by estimating the range of possible values of the substrate modulus.

We assume a monolayer of enzyme molecules of 50 Å in diameter, having a turnover number and K_M of 100 sec^{-1} and 10^{-4} M, respectively. Then the saturation rate per unit area is 10^{-9} mol cm^{-2} sec^{-1}, in agreement with previous calculations (Shuler *et al.*, 1973). If the substrate has to diffuse a distance of 100 nm through a stagnant liquid, in which the diffusivity is 10^{-6} cm^2 sec^{-1}, the value of h_s is calculated as 0.1 cm sec^{-1}.

These data yield a value of 10^{-4} for μ. With the above quantities which are representative of actual physiological conditions, it appears that there is no diffusional inhibition due to the slowness of substrate diffusion in the aqueous cellular fluid, if the diffusivity is greater than 10^{-9} cm^2 sec^{-1}.

Nevertheless external diffusion limitations can play a significant role when the substrate has to diffuse across a membrane to the bound enzyme. Literature data (Davson and Danielli, 1952) indicate that in biological membranes the permeability coefficients of undissociated small molecules range from 10^{-6} to 10^{-12} cm sec^{-1} and the permeability of large and ionized molecules is even smaller. Since the permeability coefficient is equivalent to h_s, the transport coefficient used in this study, the previous set of kinetic parameters yields $\mu = 1$ for $h_s = 10^{-5}$ cm sec^{-1}. As the membrane permeability coefficient is often several orders of magnitude smaller than 10^{-5} cm sec^{-1}, the actual value of μ can be much greater than unity. Therefore, diffusional inhibition is indeed a factor to be considered in cellular physiology.

The results of the theoretical analysis clearly demonstrate that great caution has to be exercised when the effect of an inhibitor on an enzyme in its natural environment is inferred from experiments with the enzyme in free solution. Furthermore they also suggest that consideration be given to the possible interaction of diffusional and chemical inhibition in studies concerned with the regulatory mechanisms of metabolic processes.

Appendix I

Micro- and Macroenvironment of the Bound Enzyme. When an enzymic reaction takes place in a well-stirred homogeneous solution the concentration of all species is uniform throughout the system. This is generally not the case when the enzyme is bound to a solid structure, such as a membrane.

First, ion–ion, polar, and nonpolar interactions of the various species with the enzyme support may lead to nonuniform concentration. Second, concentration gradients may be established by the relatively slow diffusion of the reactants and products to and from the bound enzyme, respectively. As a result, the microenvironment, *i.e.*, the immediate vicinity of the bound enzyme, may have a different composition with respect to those species which influence the rate of reaction than that of the system at a certain distance away from the bound enzyme, *i.e.*, the macroenvironment.

When the behavior of heterogeneous enzyme systems is investigated *in vitro* the stirred aqueous solution containing the bound enzyme constitutes the macroenvironment. Although the enzyme activity is determined by the local concentrations of the different species in the microenvironment, the respective concentrations are usually measured in the bulk solution, *i.e.*, in the macroenvironment. On the other hand, in the cellular milieu the substrate may have to diffuse across a membrane to the bound enzyme. Then the substrate concentration in the microenvironment can be different from that in the macroenvironment, *i.e.*, at the other side of the membrane which is exposed to the source of the substrate.

In the present study the effect of diffusion resistances in the surrounding solution or membrane on the rate of the enzymic reaction at steady state is investigated. Therefore concentration differences between the micro- and macroenvironment have to be considered only for those species which are produced or consumed in the reaction.

Appendix II

Effective and Inherent Rates. The transport of the substrate from the macro- to the microenvironment and the consumption of the substrate in the reaction catalyzed by a surface bound enzyme take place consecutively and, at steady state, proceed at the same rate. This effective rate of the reaction depends both on the mass transport coefficient for the substrate, h_s, and the kinetic parameters of the reaction, V_{max} and K_M. The effective rate is usually more strongly influenced by the parameters of one process than by those of the other. In order to interpret this dependence it is convenient to define two inherent rates characteristic for the two steps involved. (a) The inherent rate of the reaction or the inherent enzyme activity, V_{kin}, is defined as the reaction rate which is solely determined by the kinetic parameters of the enzymic reaction and the concentration of the substrate in the macroenvironment, [S], and would be obtained if the diffusion of the substrate was infinitely fast. Thus, in the case of noncompetitive inhibition

$$V_{kin} = \frac{V_{max}[S]}{[1 + ([I]/K_I)][K_M + [S]]}$$

(b) The inherent rate of combined molecular and convective diffusion of the substrate, V_{diff}, is defined as the rate which is solely determined by the transport coefficient and the macroenvironmental concentration and would be obtained if the enzyme activity was infinitely high. Thus, $V_{diff} = h_s[S]$.

The effective rate of the surface reaction is more influenced by that process which has the lower inherent rate. In the limit, when one of the inherent rates is much smaller than the other the effective rate is practically equal to the lower inherent rate. Under such conditions, it is convenient to distinguish between kinetically controlled reaction, which proceeds with a rate equal to V_{kin}, and diffusion controlled reaction, which proceeds with a rate equal to V_{diff}. In free solution diffusional resistances can be neglected and the measured rate of enzymic reaction is equal to V_{kin}.

In heterogeneous systems, however, the possibility of diffusional resistances has always to be considered. In this study the interaction of diffusion and reaction has been quantified by the substrate modulus, μ_1, which is equal to V_{kin}/V_{diff} when [S] is smaller than K_M, that is, the enzymic reaction is first order.

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Inhibition of Bound Enzymes. II. Characterization of Product Inhibition and Accumulation†

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ABSTRACT: Diffusion resistances cause not only substrate depletion but also product accumulation in the microenvironment of bound enzymes. As a result product inhibition is more pronounced in the presence than in the absence of diffusional limitations at a given product concentration in the macroenvironment. The antienergistic interaction between chemical and diffusional inhibition, however, makes the activity of bound enzymes less sensitive to changing product concentration in the macroenvironment. The interplay between various types of product inhibition and diffusion resistances is illustrated by plots of the reaction rate against the ratio of the rate and the macroenvironmental substrate concentration. Combined diffusional and product inhibition can significantly affect the kinetic behavior of immobilized enzyme systems and the magnitude of feedback inhibition in cellular metabolism.

The inhibition of bound enzymes by a substance which is neither a substrate nor a product of the reaction has been treated in the preceding paper (Engasser and Horvath, 1974a). In this study the effect of product inhibition is investigated.

Many enzymes are inhibited by the product of the reaction and this feedback regulation is assumed to play a fundamental role in the control of biological systems. Therefore, product inhibition of enzymes in free solution has been extensively treated in the literature (Webb, 1963). In heterogeneous enzyme systems, however, diffusional resistances can give rise to both substrate depletion and product accumulation in the immediate vicinity of the enzyme. Since the degree of inhibition depends on the concentration of both the product and the substrate in the microenvironment, the product inhibition of naturally or artificially bound enzymes is more complex than that of enzymes in free solution (Goldman *et al.*, 1968a).

The following analysis encompasses the quantitative treatment of the combined effect of substrate depletion and product accumulation in the microenvironment on the kinetic behavior of bound enzymes. The results are presented in graphical form in order to facilitate the recognition of such phenomena from experimental data.

Theoretical Analysis

Product Accumulation and Inhibition. In the simplest case a heterogeneous enzyme reaction takes place at a surface covered with bound enzyme molecules. Both the substrate and the product diffuse to and from the surface through a nonreactive membrane or the surrounding liquid. The overall reaction consists of the following three steps. First the substrate is transported to the catalytic surface, *i.e.*, the microenvironment. Then the substrate is converted into product and finally the product is transported to the macroenvironment of the enzyme, which is often the bulk solution in practice.

If the reaction follows the Michaelis–Menten scheme and the product is a competitive inhibitor that only affects the K_M of the reaction, the rate of the enzymic reaction, v, is expressed by

$$v = \frac{V_{max}[S_0]}{K_M[1 + ([P_0]/K_p)] + [S_0]} \quad (1)$$

where K_p is the product inhibition constant, $[S_0]$ and $[P_0]$ are the concentrations of the substrate and product at the surface, respectively.

The rate of external transport of each species can be expressed as the product of the corresponding transport coefficient and the driving force. The latter is given by the concentration difference between the macro- and the microenvironment of the enzyme. At steady-state substrate transport, enzymatic reaction and product transport must proceed at the same rate so that

$$h_s([S] - [S_0]) = \frac{V_{max}[S_0]}{K_M[1 + ([P_0]/K_p)] + [S_0]} = h_p([P_0] - [P]) \quad (2)$$

where [S] and [P] are the concentrations in the macroenvironment, and h_s and h_p are the transport coefficients of the substrate and the product, respectively. Introducing a dimensionless substrate concentration

$$\beta = [S]/K_M \quad (3)$$

the substrate modulus without inhibition, μ, which expresses the diffusion resistance for the substrate

$$\mu = V_{max}/h_s K_M \quad (4)$$

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‡ Recipient of Yale University Fellowship.